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Lysosomal cathepsins in embryonic programmed cell death

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Abstract

During limb development, expression of *cathepsin D* and *B* genes prefigure the pattern of interdigital apoptosis including the differences between the chick and the webbed digits of the duck. Expression of *cathepsin L* is associated with advanced stages of degeneration. Analysis of *Gremlin−/−* and *Dkk−/−* mouse mutants and local treatments with BMP proteins reveal that the expression of *cathepsin B* and *D* genes is regulated by BMP signaling, a pathway responsible for triggering cell death. Further cathepsin D protein is upregulated in the preapoptotic mesenchyme before being released into the cytosol, and overexpression of *cathepsin D* induces cell death in embryonic tissues by a mechanism including mitochondrial permeabilization and nuclear translocation of AIF. Combined inhibition of cathepsin and caspases suggests a redundancy in the apoptotic molecular machinery, providing evidence for compensatory activation mechanisms in the cathepsin pathway when caspases are blocked. It is concluded that lysosomal enzymes are functionally implicated in embryonic programmed cell death.

Keywords: Interdigital cell death; Caspase; AIF; Limb development; Apoptosis

Introduction

Programmed cell death (PCD) is a genetically controlled mechanism that ensures appropriate development and maintenance of a multicellular organism. PCD occurs during development of both vertebrate and invertebrate embryos, and its deregulation causes many developmental alterations. The areas of interdigital cell death during limb development provide a very illustrative example of massive cell death, with a sculpturing function in morphogenesis (Zuzarte-Luis and Hurle, 2002), but virtually all developing organs in vertebrates also exhibit well-defined areas of cell death at precise stages of its formation.

During the last decades, the execution of programmed cell death has been proposed to occur following two main pathways, cytochrome *c* liberation from the mitochondria or activation of death receptors (Ashkenazi and Dixit, 1998; Hengartner, 2000). Both pathways lead to the activation of a cysteine proteases family known as caspases, which execute the cell death program, leading to typical morphologic changes termed apoptosis. PCD includes cell shrinkage, chromatin condensation and formation of condensed cell fragments. The involvement of caspases in cell death was initially demonstrated in *C. elegans* and lately confirmed in most apoptotic systems, including cell death during limb development (Mirkes et al., 2001; Huang and Hales, 2002; Ali-Khan and Hales, 2003; Zuzarte-Luis and Hurle, 2005). However, phenotypes caused by inhibition of embryonic cell death are scarce in mice deficient for caspases. Thus, syndactyly secondary to inhibition of interdigital cell death is absent from mice deficient in either *caspase 2*, *caspase 3*, *caspase 6*, *caspase 7*, *caspase 8* or *caspase 9* (Kuida et al., 1996; Woo et al., 1998; Wang and Lenardo, 2000), despite all these caspases being activated in dying interdigital cells (Zuzarte-Luis et al., 2006). Consistent with these findings, other loss of function experiments in mouse and chicken showed that caspase activity is involved but dispensable for cell death during development (Chautan et al., 1999; Oppenheim et al., 2001; Yaginuma et al., 2001). All these facts prompted us to explore alternative and/or complementary mechanisms responsible for embryonic cell death.

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The involvement of lysosomal activation in PCD was already proposed some decades ago (Hurle and Hinchcliffe, 1978), although poor attention was dedicated to this finding afterwards. There is now growing evidence suggesting that other proteases, including the lysosomal components (as cathepsins), are implicated in some apoptotic processes rather than being responsible for unspecific degradation of apoptotic cells upon phagocytosis (Borner and Monney, 1999; Stoka et al., 2001; Boya et al., 2003; Guicciardi et al., 2004). Thus, alternative modes of PCD with specific morphological characteristics at the cellular level have been proposed and named as apoptosis-like PCD and necrosis-like PCD (Leist and Jaattela, 2001a). The cysteine proteases cathepsins B and L and the aspartatic protease cathepsin D are the most abundant proteases of the lysosomes and are good candidates to account for cell degradation during PCD (Leist and Jaattela, 2001b). Several reports suggest that lysosomal proteases favor the release of mitochondrial factors to the cytoplasm, and indeed cathepsin D has been reported to trigger caspase-independent AIF (apoptosis-inducing factor) release in T lymphocytes committed to die (Bidere et al., 2003). AIF is a mitochondrial factor that, once released to the cytoplasm, is translocated into the nucleus, thereby triggering chromatin condensation and DNA degradation, even in the absence of caspase activation (Susin et al., 1999; Loeffler et al., 2001). Additionally, cathepsins are able to activate caspases, indicating that they can act in the early steps of apoptosis (Ishizaki et al., 1998; Schotte et al., 1998; Vancompernolle et al., 1998).

In this work we report a possible role for cathepsins D, B and L in programmed cell death during embryogenesis. We describe the expression of the *cathepsin D* gene during cell death in many tissues of the developing embryo, and using the interdigital tissue as an experimental model system, we show that this protease is activated and released from the lysosomes in preapoptotic cells. Furthermore, we provide evidence for a cooperation of cathepsin D with caspases in the degradation of interdigital mesenchymal cells.

Materials and methods

Animal models

We employed Rhode Island chick embryos ranging from 3 to 9 days of incubation (stages 20–35, Hamburger and Hamilton, 1951), Royal Peking duck embryos ranging from day 8 to day 11 of incubation and wild type mouse or *Dkk1−/−* (Mukhopadhyay et al., 2001) or *Gremlin−/−* (Khokha et al., 2003) mouse mutants from 10 to 14 dpc.

Probes and in situ hybridization

Fragments of chicken *cathepsin D* (cCatD), *cathepsin B* (cCatB) and mouse *cathepsin D* (mCatD), *cathepsin B* (mCatB) and *cathepsin L* (mCatL) genes were obtained by RT-PCR. First-strand cDNA was synthesized with random hexamers and M-MulV reverse transcriptase (Fermentas) and 1 mg of total RNA from day 7 autopods. The following primers were used for subsequent PCR amplification; for cCatD—fwd: 5′-TTC TGC GCT TCT GCT TTA GGG-3′ and rev: 5′-TGA GTG GGT TTC TAA TCC TGA-3′; for cCatB—fwd: 5′-ACT GAC ATG AGC TAT GTG AAG-3′ and rev: 5′-GTA GGA TGT GAT GCC GTA GT-3′; for mCatD—fwd: 5′-TAA TGC TTG GTG GCA CTG ACT-3′ and rev: 5′-TCT GGG TCA GAG CAG GTT TCT-3′; for mCatB—fwd: 5′-GAC GCA ACT TCT ACA ATG TTG-3′ and rev: 5′-TAG ATT TCT GCC ATG ATC TC-3′; and mCatL—fwd: 5′-GAC TGT ATG GCA CGA ATG AG-3′ and rev: 5′-TGT CAT TAG CCA CAG CGA AC-3′. Chicken *cathepsin L* (cCatL) was obtained from the cDNA library described in Zuzarte-Luis et al. (2004).

The PCR conditions were 94°C, 4 min and then 35 cycles of 94°C, 20 s; 50°C, 30 s; 72°C, 60 s; and final extension at 72°C, 10 min. PCR products were subcloned into pGEM T-easy (Promega). Chicken and mouse cathepsins digoxygenin-labeled sense and antisense RNA probes were generated for in situ hybridization analysis. In situ hybridization was performed in whole mount specimens of limb buds as described in Zuzarte-Luis et al. (2004) and in interdigital cell dissociates as described in Zuzarte-Luis et al. (2006). For double labeling purposes, the in situ hybridization was performed first followed by the immunolabeling or the TUNEL assay.

Antibodies and immunolabeling

The following primary antibodies were employed: polyclonal antibodies against AIF, cathepsins B, D and L (Santa Cruz Biotechnology), histone H4 acetylated (Chemicon) and monoclonal antibody against lysosomes (LEP100) and macrophages (TAP1) from the Developmental Studies Hybridoma Bank.

For double labeling purposes, we first performed the immunolabeling followed by the TUNEL assay. Immunolabeling in cell dissociates was performed as described in Zuzarte-Luis et al. (2006).

TUNEL analysis of dying cells

Interdigital cells dissociate and paraffin sections of cultured autopods were analyzed for apoptotic DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP-TRIC nick end labeling (TUNEL) assay, using the in situ cell death detection kit (Roche). In both cases, the manufacturer's instructions were followed.

Acridine orange or neutral red vital stains were employed as an alternative to the TUNEL method in whole mount autopod cell death detection.

Confocal microscopy

Cell samples were examined with a laser confocal microscope (BioRad, MRC 1024 and Zeiss LSM510) by using a plan-apochromatic 63× oil objective (1.4 NA) and argon ion laser (488 nm) to excite FITC fluorescence and a HeNe laser (543 nm) to excite Texas Red. For double labeling experiments, images of the same confocal plane were sequentially recorded and pseudocolor images were generated and superimposed. TIFF (RGB) images were transferred to Adobe Photoshop 7 software (Adobe Systems Inc) for presentation. All images presented within each figure were identically adjusted for contrast, brightness and dynamic resolution.

Experimental manipulation of the limb

The regulation of the *cathepsin D* gene by BMPs was studied by analyzing the effects of local administration of recombinant BMP7 (a gift of Creative Biomolecules, Hopkinton, MA) into the limb mesoderm, at concentrations ranging from 10 to 100 μg/ml using heparin acrylic beads as carriers (Sigma) for 12 h, 18 h and 20 h. After the period of incubation, samples were processed for in situ hybridization. In all the experiments, PBS soaked beads were also implanted as a control.

The in vivo cathepsin D and caspase inhibition experiment was performed by microinjection of a solution composed of 1 mM Z-VAD-FMK, 1.25 mM pepstatin A and 0.2% Fast Green in the third interdigit of 7.5-day chicken embryos. The embryos were allowed to develop for a period of 24 h or 48 h after which limb phenotype was analyzed.

Western blot

For western blot analysis, total protein from the interdigits of 6.5-day, 7-day and 7.5-day embryos were obtained by lysis with RIPA buffer (150 mM NaCl, 1,5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% TritonX-100, 0.1% SDS, 1% deoxicolate, 50 mM HEPES, pH 7.4) supplemented with the

protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin (10 mg/ml) and aprotinin (10 mg/ml), for 15 min on ice, followed by centrifugation at 13,200 × *g* for 10 min at 4°C.

Protein concentration was determined by the Bradford technique. For all western blots, 50 mg of the isolated proteins was used. Each western blot was repeated three times, and for all blots α-tubulin (Sigma) was used as load control. All western blots were quantified using "Scion Image".

Autopodial explants culture

Autopods of embryos at day 6 or 6.5 of incubation were microsurgically explanted and cultured in DMEM (GIBCO) supplemented with 1% L-glutamine (Cambrex), 5% chicken serum (Sigma), 10% fetal bovine serum (GIBCO) and 1% PenStrep antibiotic (Sigma). Experimental autopods were cultured in the presence of Z-VAD-FMK (concentrations ranging from 50 to 100 μM; Promega) and/or pepstatin A (concentrations ranging from 25 to 100 μM; Sigma) for 12 h, 24 h and 48 h. After this period, the tissue was processed for whole mount acridine orange staining, TUNEL assay in paraffin sections or western blot.

Cathepsin D overexpression experiments

The full-length *cathepsin D* gene was cloned into the pCX-IG (IRES-GFP; Niwa et al., 1991) vector, and overexpression was performed in limb mesodermal cells and a chicken fibroblast cell line (DF1). The vector pCX-EGFP was used as control.

Embryonic limb mesenchymal cell culture obtained from leg buds of day 4 of incubation and chicken fibroblasts were cultured in DMEM (GIBCO) supplemented with 1% L-glutamine (Cambrex), 2% chicken serum (Sigma), 10% fetal bovine serum (GIBCO) and 1% PenStrep antibiotic (Sigma). Cells were transfected with Fugene 6 (Roche) according to the manufacturer's instruction and incubated for 24 h, 48 h and 72 h and then fixed in 4% paraformaldehyde for 10 min and processed for immunolabeling and TUNEL assay.

The number of dead cells in the culture medium was counted using trypan blue staining 24 h, 48 h and 72 h upon pCX-IG-cathepsin D transfection. Statistical analysis was carried out by ANOVA followed by Bonferroni's test (GraphPAD InStat software). A p value <0.05 was considered to be statistically significant. The results are expressed as mean ± standard error of values from a minimum of four individuals per group.

For in vivo neural tube electroporation, eggs were windowed at day 2 of incubation, the DNA was microinjected in the lumen of the neural tube and the right side of the NT was electroporated (4 electric pulses of 13 V of 20 ms, with an interval of 30 ms). The pCX-EGFP was electroporated as a control. Embryos were harvested 24 h and 48 h after electroporation and were processed for in situ hybridization, immunolabeling and TUNEL assay.

Results

Cathepsin D is expressed in the interdigital mesoderm of the chick leg bud committed to die

Cathepsin D is expressed at low levels in the chicken limb mesoderm throughout the whole period under study. In addition, well-defined domains of high expression were detected in a pattern that correlates with the patterns of limb cell death (Fig. 1A). In the interdigital tissue, *cathepsin D* exhibits a pattern of expression prefiguring the pattern of interdigital cell death, appearing first in the most proximal part of the interdigits and extending distally as development takes place (Fig. 1B). This correlation between *cathepsin D* gene expression and interdigital cell death was also observed in the webbed digits of duck embryos in which cell death is restricted to the most distal portion of the interdigital tissue (Figs. 1C, D).

Cathepsin D expression appeared also as a precise marker for the anterior and posterior areas of limb cell death (ANZ and PNZ; Figs. 1E, F) and also in many other areas of cell death present in different developing organs including lens, heart and somites (not shown). However, neither the areas of cell death present in the developing interphalangeal joints (Figs. 1G, H) nor the area of cell death located between the skeletal elements of the zeugopod, termed the opaque patch (not shown), was positive for *cathepsin D* expression. These findings point to differences in the death machinery among different areas of embryonic cell death.

By immunocytochemistry, we observed that interdigital mesenchymal cells showed a high level of expression of cathepsin D inside the lysosomes at stages immediately preceding cell death (Fig. 2A). It is important to note that cathepsin D expression could correlate with abundance of macrophages within the mesenchymal tissue. In this apoptotic system, phagocytes are migratory cells originated from the hematopoietic system instead of being locally generated (Cuadros et al., 1992; Hopkinson-Woolley et al., 1994). Hence, to discard the possibility that the interdigital domains of cathepsin D reflected the activity of incoming macrophages, we analyzed in interdigital tissue dissociates the expression of cathepsin D at RNA and protein levels in combination with TUNEL or with TAP1, a monoclonal antibody specific for macrophages (Guillemot et al., 1984), respectively. By in situ hybridization, we observed that *cathepsin D* transcripts were present in most interdigital mesenchymal cells, including those at initial stages of apoptosis detected by incipient TUNEL labeling (Fig. 2B). In addition, as shown in Fig. 2C, cathepsin D protein was present in macrophages that were TAP1 positive but also in almost all interdigital mesodermal cells negative for TAP1.

Synthesis, activation and lysosomal release of Cathepsin D precede programmed cell death

We have analyzed the expression of cathepsin D in the interdigital tissue by western blot and we found that zymogen and active protein are present at day 5.5 of incubation, before the onset of cell death, when *cathepsin D* gene is expressed at basal levels through the limb mesenchyme. Interestingly, by day 6.5 of incubation, we have detected a significant increase in cathepsin D protein, including the zymogen and its active form (Fig. 3A). This upregulation appears at the beginning of the cell death program (see Garcia-Martinez et al., 1993; Zuzarte-Luis et al., 2006) and suggests an active role for cathepsin D in programmed cell death in the interdigital tissue. However, the functional implication of cathepsin D in the death process would require the release of this enzyme from the lysosome. To investigate this aspect, we analyzed by confocal microscopy the distribution of cathepsin D in combination with a lysosomal marker (LEP1000; Figs. 3B–G). We observed that, at the beginning of the dying process, all mesenchymal cells were positive for lysosomal cathepsin D

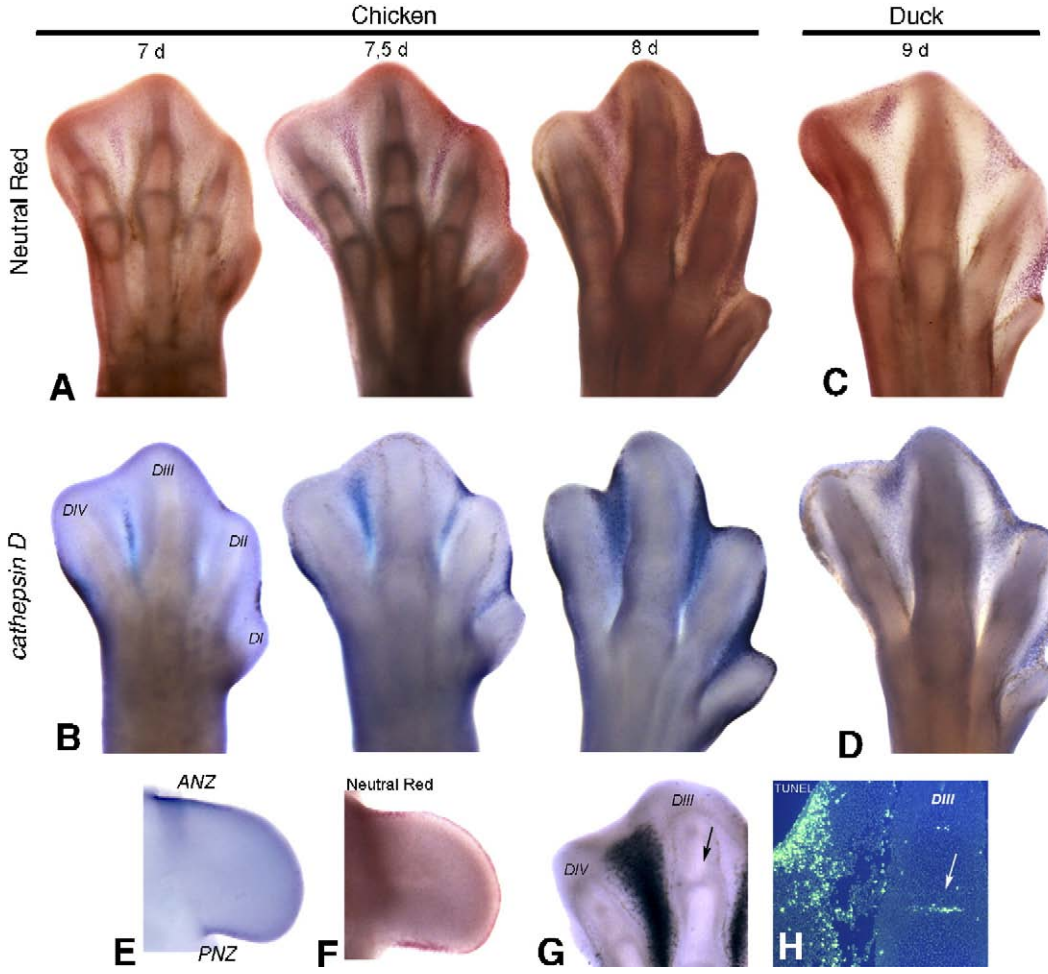

Fig. 1. Correlation between *cathepsin D* expression and patterns of cell death in chick and duck embryos. (A) Autopods of chicken embryos of days 7, 7.5 and 8 of incubation stained with neutral red showing the sequence of interdigital cell death. (B) Whole mount in situ hybridizations showing the pattern of expression of the *cathepsin D* gene in the same stages of incubation as in panel A. (C, D) Duck leg buds at day 9 of incubation stained with neutral red (C) and hybridized with *cathepsin D* probe (D). Note the restricted distal expression in the second and third interdigits of the duck (D) in contrast to the widespread interdigital distribution in the 8-day chick autopod (B). (E) *Cathepsin D* expression in the anterior and posterior margin of the chick limb bud at day 3.5 of incubation in correlation with ANZ and PNZ evidenced by neutral red staining in panel F. (G) Expression of *cathepsin D* in a longitudinal vibratome section of the leg bud at day 7.5 of incubation showing a total absence of transcripts in the developing interphalangeal joints (black arrow). (H) TUNEL staining in paraffin section of a 7.5-day leg bud evidencing the presence of apoptotic cells in the interphalangeal joints (white arrow).

labeling (Figs. 3B–D), while a significant number of mesenchymal cells (7.3±1.3%) showed intense cytoplasmic labeling for cathepsin D out of the lysosomes (Figs. 3E–G). This percentage is considerably high taking into account that at this stage the rate of cell death estimated by flow cytometry is 34.74% (Zuzarte-Luis et al., 2006; Figs. 3E–G).

Regulation of cathepsin D expression by BMP signaling in avian and mouse limb buds

Since interdigital cell death has been found to be under the control of BMPs, we analyzed changes in the expression of *cathepsin D* in the chick leg bud following interdigital implantation of beads bearing BMP-7 protein (Macias et al., 1997). Consistent with a role for cathepsin D in the onset of apoptosis, we observed an initial upregulation of *cathepsin D* gene expression 10 h after the treatment which then increased dramatically in parallel to, but preceding, the establishment of the area of cell death triggered by the BMP bead (Figs. 4A–B).

To further investigate the relationship between *cathepsin D* expression and BMP-regulated cell death, we extended our study to mice. We first observed that *cathepsin D* expression in the mouse was also a precise marker of limb programmed cell death, including the areas of interdigital cell death (Fig. 4C). We then selected two of mouse mutants with alterations in the BMP signaling pathway that exhibit cell death phenotypes to analyze *cathepsin D* gene regulation.

The *Dickkopf-1* (*Dkk*) gene is a negative modulator of the Wnt pathway involved in the control of interdigital cell death (Grotewold and Ruther, 2002). It has been shown that interdigital expression of *Dkk* is under the control of the BMP signaling pathway responsible for limb apoptosis, and *Dkk−/−* mice exhibit syndactyly both in fore and hind limbs, affecting mainly digits 2–3 (Mukhopadhyay et al., 2001). Here we show

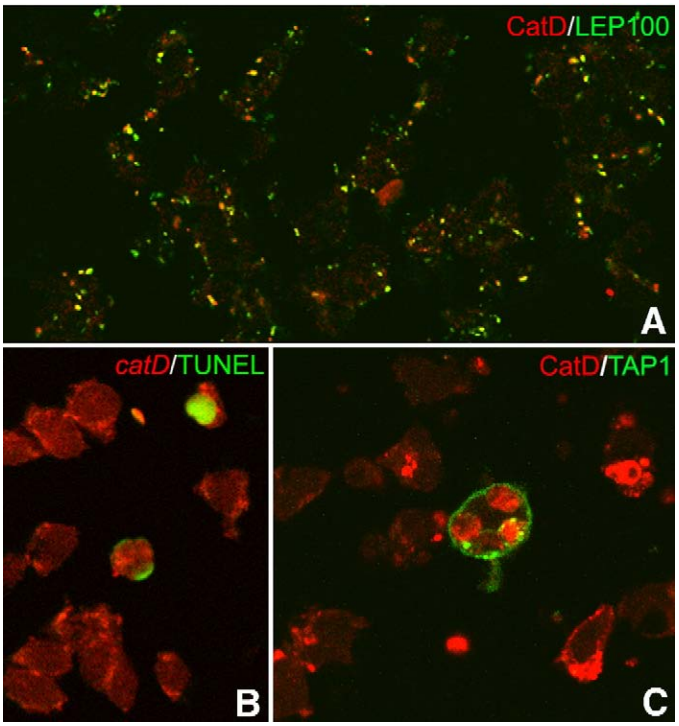

Fig. 2. Cathepsin D protein is found not only in invasive macrophages but also in the interdigital preapoptotic mesenchymal cells. (A–C) Confocal images showing cellular dissociates from the interdigital tissue of 7.5-day embryos. (A) Immunodetection of lysosomes (green) and cathepsin D (red) in preapoptotic interdigital mesenchymal cells. (B) TUNEL staining of apoptotic programmed cell death (green) combined with *cathepsin D* mRNA expression (red) revealed by in situ hybridization. Staining shows that preapoptotic and apoptotic cells are overloaded of *cathepsin D* transcripts. (C) Cathepsin D (red) and macrophage marker TAP1 (green) immunostaining shows that most of the cathepsin D expressing cells correspond to preapoptotic mesenchymal interdigital cells, although macrophages are also overloaded.

that this mutant has a significant reduction of the interdigital expression domain of *cathepsin D* prefiguring the syndactylous phenotype (Fig. 4D).

Gremlin is a BMP antagonist that promotes limb outgrowth by negatively controlling the apoptotic promoting effect of BMPs (Merino et al., 1999). It has been shown that *Gremlin−/−* mice exhibit severe limb defects caused by increased apoptosis (Khokha et al., 2003; Michos et al., 2004). Consistent with this phenotype, we detected an abnormal domain of the *cathepsin D* gene in the anterior limb mesoderm at day 10.5–11.5 dpc that is absent in wild type embryos (Figs. 4E–F). This domain is coincident with the zone of increased cell death described in this mutant (Michos et al., 2004).

Cathepsin D cooperates with caspases in mediating programmed cell death

To evaluate the role of cathepsin D in interdigital cell death, we performed a variety of experimental approaches using pepstatin A to inhibit cathepsin function. Pepstatin A is a specific inhibitor of cathepsin D largely used for loss of function experiments. In initial experimental approaches, we observed that interdigital cell death was almost unaffected in autopods cultured in the presence of pepstatin A for 12 h or 24 h (Figs. 5B, F) when compared to the controls (Figs. 5A, E). We interpreted these results as indicative of a redundancy in the molecular machinery responsible for the execution of the death program. According to this interpretation, it was likely that, under our experimental conditions, caspases or other apoptotic executors (i.e. other cathepsins) might substitute the function of cathepsin D. To check this possibility, autopodial explants obtained from embryos at day 6.5 of incubation were cultured for 12 h, 24 h or 48 h in the presence of the pan-caspase inhibitor Z-VAD-FMK with or without pepstatin A. As previously reported (Chautan et al., 1999; Zuzarte-Luis et al., 2006), cell death was only moderately reduced upon addition of the pan-caspase inhibitor to the culture medium (Figs. 5C, G). However, when Z-VAD-FMK was added in combination with pepstatin A, attenuation of cell death was significant (Figs. 5D, H), suggesting that caspases and cathepsin D may cooperate in the elimination of the interdigital cells. Furthermore, by analyzing the levels of cathepsin D in the interdigital tissue cultured in the presence of Z-VAD-FMK, we observed that this protease is still released from the lysosomes in most preapoptotic cells, and, most importantly, we found a significant increase in the amount of cathepsin D after the treatment, with respect to control limbs (Fig. 5I). Together, these findings suggest that the execution of the cell death program is ensured by different mechanisms, including at least cathepsin D and caspases, but most likely also involving other degradation pathways.

Interdigital microinjection experiments confirmed, in vivo, the results obtained for autopodial explants cultures. Thus, regression of interdigital tissue was inhibited or delayed (20 out

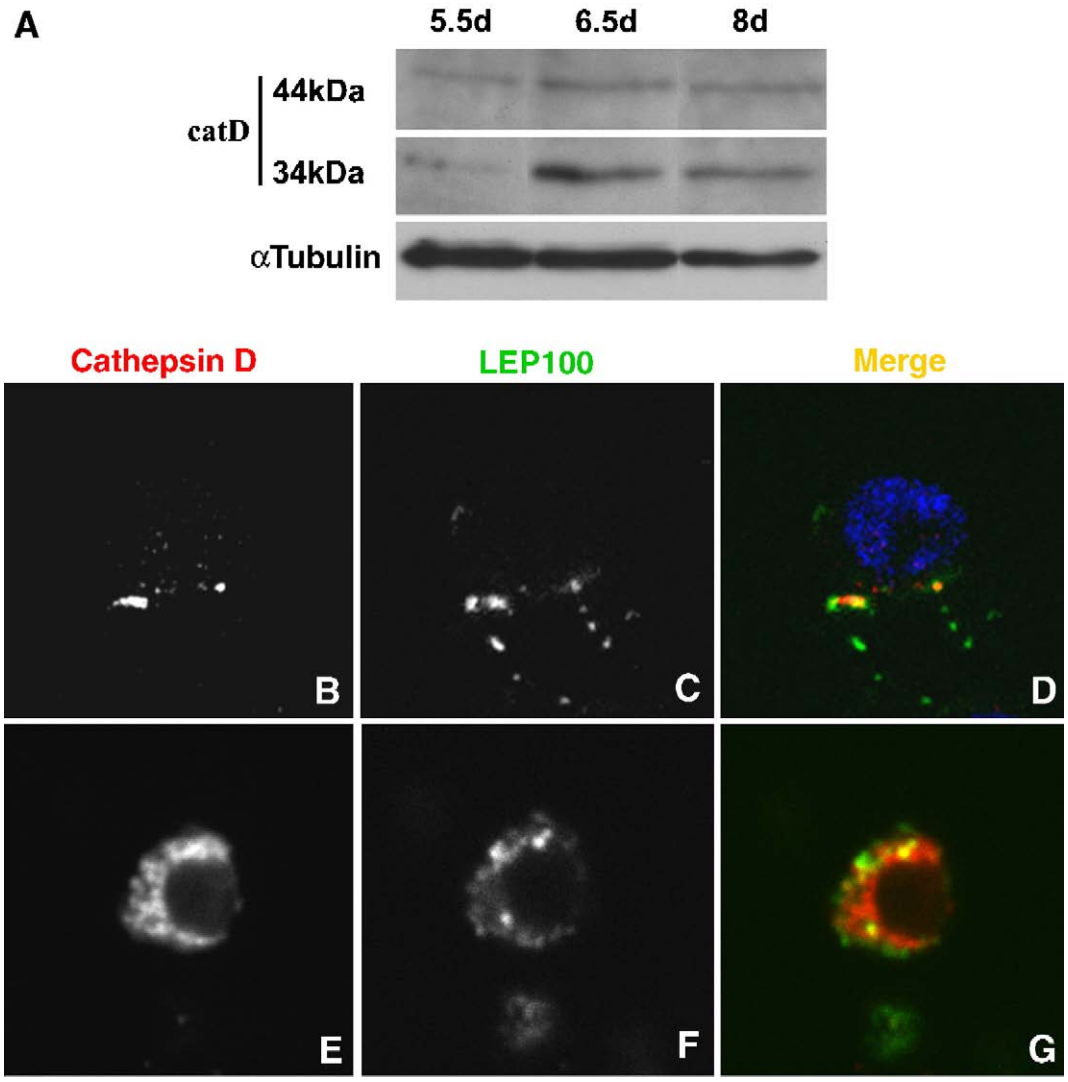

Fig. 3. Activation and lysosomal release of cathepsin D in the interdigital mesoderm. (A) Western blot showing the increase of total cathepsin D zymogen (44 kDa) and active cathepsin D (34 kDa) in the interdigits at days 5.5, 6.5 and 8 of incubation showing an increase in the expression from pre to the apoptotic stages (α-tubulin was used as loading control). (B–G) Confocal images showing interdigit mesodermal cells triple immunolabeled for cathepsin D (red), lysosomal membrane protein (LEP100; green) and histone H4 (blue) in the interdigital mesenchyme at day 7.5 of incubation. Panels B–D illustrate that cathepsin D is restricted to the lysosomes in a healthy mesenchymal cell (positive for H4).Panels E–G illustrate that cathepsin D appears to be released into the cytosol in a dying cell (negative for H4).

of 31 experiments) by microinjection of a solution containing both inhibitors pepstatin A and Z-VAD-FMK (Figs. 5J, K), while in embryos microinjected with either pepstatin A ($n=13$) or Z-VAD-FMK ($n=12$), interdigital tissue regression was normal (not shown).

Overexpression of the cathepsin D promotes cell death in limb mesoderm and in neuroepithelial tissue

Since inhibition of cathepsin D by pepstatin A was not enough to inhibit interdigital cell death and inhibition of caspases is followed by strong promotion of cathepsin D expression preceding cell death, the potential apoptotic effect of this enzyme was explored in primary cultures of limb mesodermal cells and in a chicken fibroblast cell line. Cells were transfected with pCX-EGFP vector or pCX-*cathepsin D*-IG and cultured from 24 h to 72 h. Results described below were identical in both cell lines. We quantified the number of dead cells in these cultures and observed that cells transfected with cathepsin D gene underwent massive cell death within the 24 and 48 h of culture, in contrast with control and untransfected cultures (Fig. 6A).

Analysis of transfected cells with confocal microscopy revealed dramatic morphological changes in cathepsin D overexpressing cells. Control cultures transfected only with the GFP expressing vector exhibited a characteristic elongated morphology through the 3 days of culture (Fig. 6B). However, cells transfected with pCX-*cathepsin D*-IG vector exhibited a progressive degenerative process clearly detectable by the second day of culture (Figs. 6C–E). Prior to degeneration, elongated cells exhibited a moderate cathepsin labeling in a pattern consistent with its localization into the lysosomes or the Golgi apparatus (Fig. 6C). In contrast, cytoplasmic cathepsin D appeared progressively increased in parallel with loss of the

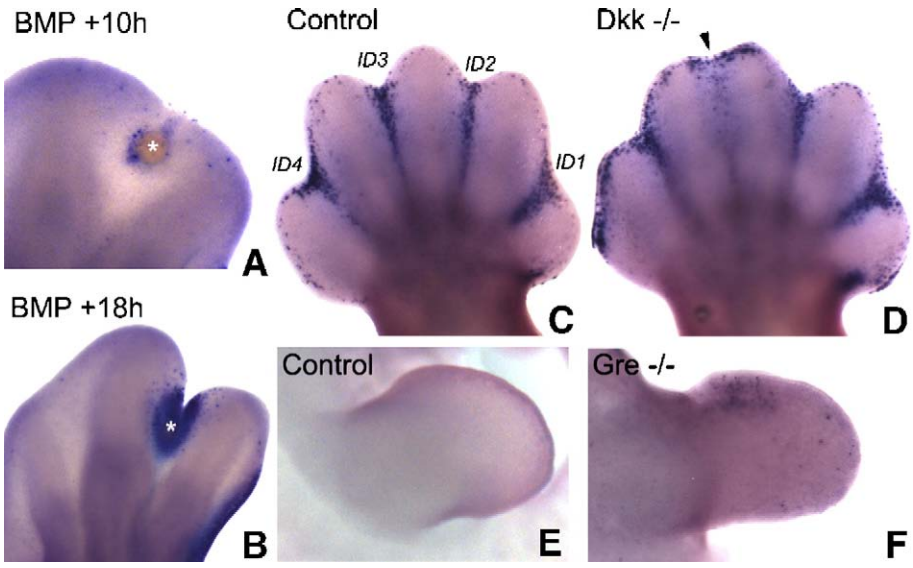

Fig. 4. Regulation of interdigital expression of *cathepsin D* in chick and mouse embryos. Panels A and B illustrate the upregulation of *cathepsin D* expression in chicken limbs 10 h (A) and 18 h (B) after the implantation of a BMP bead (asterisks). Panels C and D show the interdigital expression of *cathepsin D* in wild type (C) and *Dkk−/−* (D) mouse embryos at day 13.5 dpc. Note the intense downregulation of *cathepsin D* expression in the third interdigit of *Dkk−/−* (arrowhead). E and F show the pattern of expression of *cathepsin D* in the limb bud of wild type (E) and *Gremlin −/−* (F) embryos at day 11.5 dpc. Note the absence of expression in the wild type limb in contrast with the expression in the anterior half of the *Gremlin −/−* limb.

elongated shape (Figs. 6D–E). Finally, when cells became rounded, labeling appeared very intense and displaying a diffuse pattern of distribution indicative of a cytosolic location of the enzyme (Fig. 6E). Consistent with these findings, cells at advanced stages of degeneration exhibited an irregular loss of GFP labeling preferentially in zones of high accumulation of cathepsin D (Figs. 6F–H).

We further confirmed that cells that had lost the elongated phenotype were TUNEL positive (Figs. 7A–C) and exhibited nuclear localization of AIF, a mitochondrial apoptotic factor that is translocated into the nucleus upon triggering of the death signal (Figs. 7D–F).

Taking into account that we have observed that *cathepsin D* is expressed in most areas of embryonic cell death (including a variety of tissues and organs; not shown), we performed gain-of-function experiment to check the ability of this lysosomal enzyme to induce apoptosis in other developing tissues. For this purpose, we overexpressed *cathepsin D* by electroporation in the right half of the neural tube of 2 days chicken embryos, and as shown in Fig. 8, a significant number of TUNEL positive cells were induced in the neuroepithelial cells and in its neighbor somitic mesoderm 24 h after electroporation. In contrast, embryos electroporated with the control GFP expressing vector lacked apoptotic cells (not shown). These results indicate that cathepsin D may also be implicated in other embryonic apoptotic processes.

Cathepsins B and L are upregulated in the areas of interdigital cell death

The results described above suggest an implication of cathepsin D in embryonic cell death. However, taking into account that activation of cathepsin D requires previously lysosomal permeabilization and that cathepsin D deficient mice lack a syndactylous phenotype, it is likely that other lysosomal enzymes might also be implicated in embryonic cell death. Indeed, this was the case. By in situ hybridization, we observed that *cathepsin B* and *cathepsin L* genes also exhibit specific domains of expression in the regressing interdigital tissue of chick (Figs. 9A–B and D–F respectively) and mouse (Fig. 9I) embryos. Expression of *cathepsin B* (Figs. 9A–B) was almost identical to that of *cathepsin D* and was also positively regulated by BMPs in a similar manner (Fig. 9C). However, interdigital expression of *cathepsin L* was only detectable at advanced stages of interdigital tissue regression (Figs. 9D–F), and it was not upregulated during the first 20 h after implantation of BMP beads (not shown). These findings suggest that cathepsin L function may be associated with the phagocytic process rather than controlling apoptosis. Double immunolabeling for the macrophage marker TAP1 and cathepsin B or L revealed that both proteins were present in preapoptotic cells and in macrophages (Figs. 9G, H). Together, these findings indicate that cathepsin D and B, and perhaps cathepsin L are redundant players during mouse and chicken interdigital cell death. This finding could explain the absence of the interdigital phenotype in mice singly deficient for cathepsin D, B or L (Saftig et al., 1995; Deussing et al., 1998; Roth et al., 2000).

Discussion

The areas of cell death in the embryo were formerly described as necrotic areas, and lysosomes were considered the main death effectors. In support of this interpretation, the occurrence of lysosomal activation in different areas of programmed cell death was reported using acid phosphatases

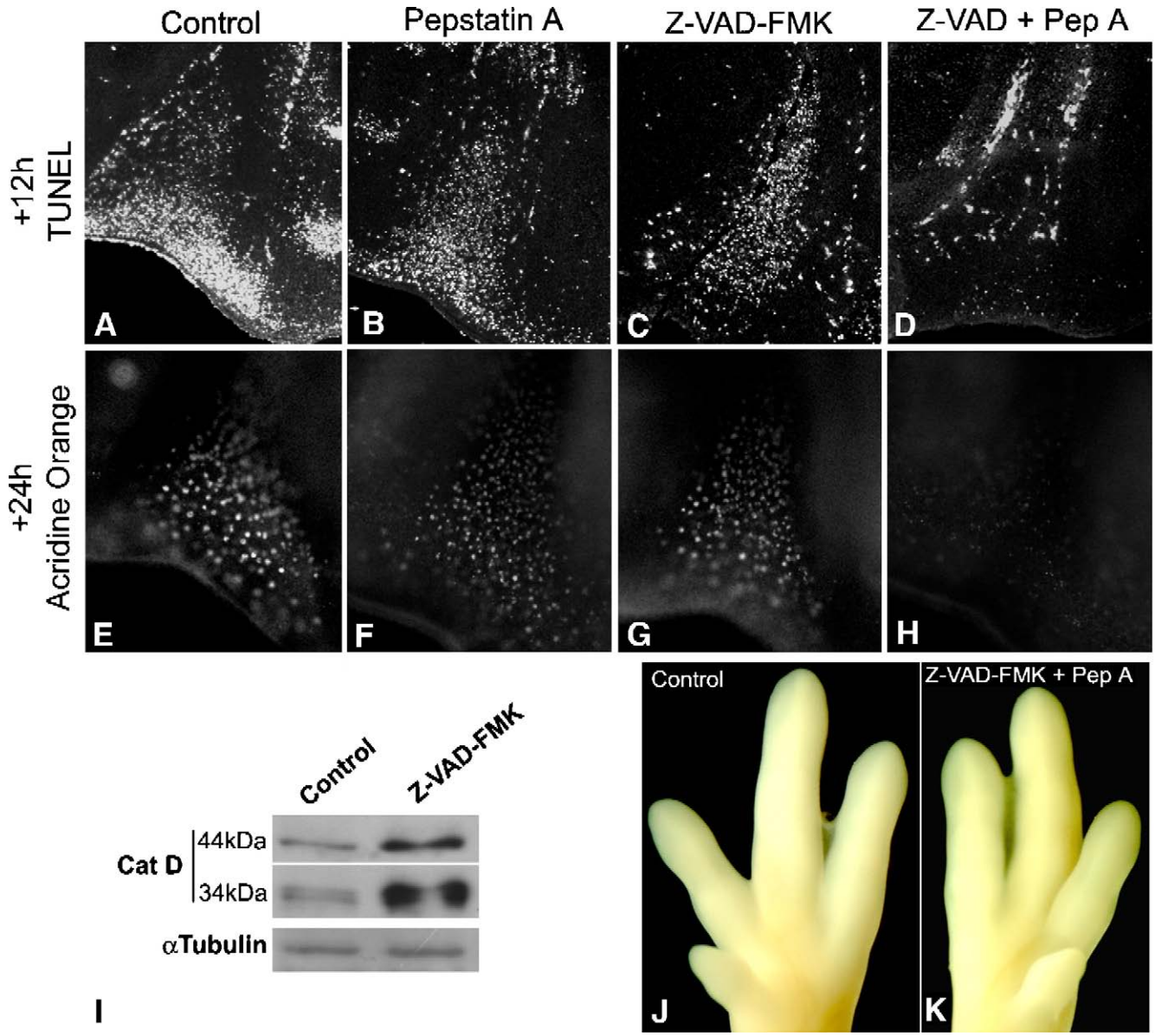

Fig. 5. Effects of caspases and/or Cathepsin D inhibition on interdigital cell death. TUNEL (A–D) and acridine orange (E–H) staining of cell death in the third interdigit of developing limbs after 12 h (A–D) and 24 h (E–H) of culture. Untreated limbs are shown in panels A and E; pepstatin A treated in panels B and F; Z-VAD-FMK treated in panels C and G; and pepstatin A and Z-VAD-FMK treated in panels D and H. Note that inhibition of cell death is only consistent following combined treatment with Z-VAD-FMK and pepstatin A (D and H). (I) Western blot of control and Z-VAD-FMK treated interdigits, showing the increase of total cathepsin D zymogen (44 kDa) and active cathepsin D (34 kDa) in the interdigits treated with the caspase inhibitor. (J, K) In vivo treatments with Z-VAD-FMK and pepstatin A. Note the delayed interdigital tissue regression in the experimental limb (K) 24 h after the treatment, in comparison with the control limb (J).

or aryl-sulphatases as cytochemical markers for electron microscopy (Hurle and Hinchcliffe, 1978). However, with the acceptance of the term apoptosis to define programmed cell death, the involvement of lysosomes in embryonic cell death was discarded. As initially proposed, apoptosis was defined as a specific death process caused by internucleosomal DNA fragmentation occurring without the participation of lysosomes (Kerr et al., 1972). This concept was strongly reinforced with the initial identification in *C. elegans* of *ced-3*, a gene encoding for a cysteine protease, as responsible for the execution of the death program (Yuan et al., 1993). Later, members of the same family of proteases, which were termed caspases, appeared to be major mediators of programmed cell death in vertebrates (see review by Jacobson et al., 1997). In *C. elegans*, *ced-3* loss of function caused inhibition of cell death. However, phenotypes resulting from inhibition of programmed cell death are scarce in mice deficient in most of the identified caspases. In fact, none of the mice deficient for caspases exhibited syndactylous limbs reflecting an inhibition of interdigital cell death (Wang and Lenardo, 2000). These findings were largely explained by the redundancy of caspases in vertebrates. However, here we confirm previous studies (Chautan et al., 1999; Zuzarte-Luis et al., 2006) showing only a very mild inhibition of interdigital cell death in autopodial explants cultured in the presence of Z-VAD-FMK to inhibit caspase function.

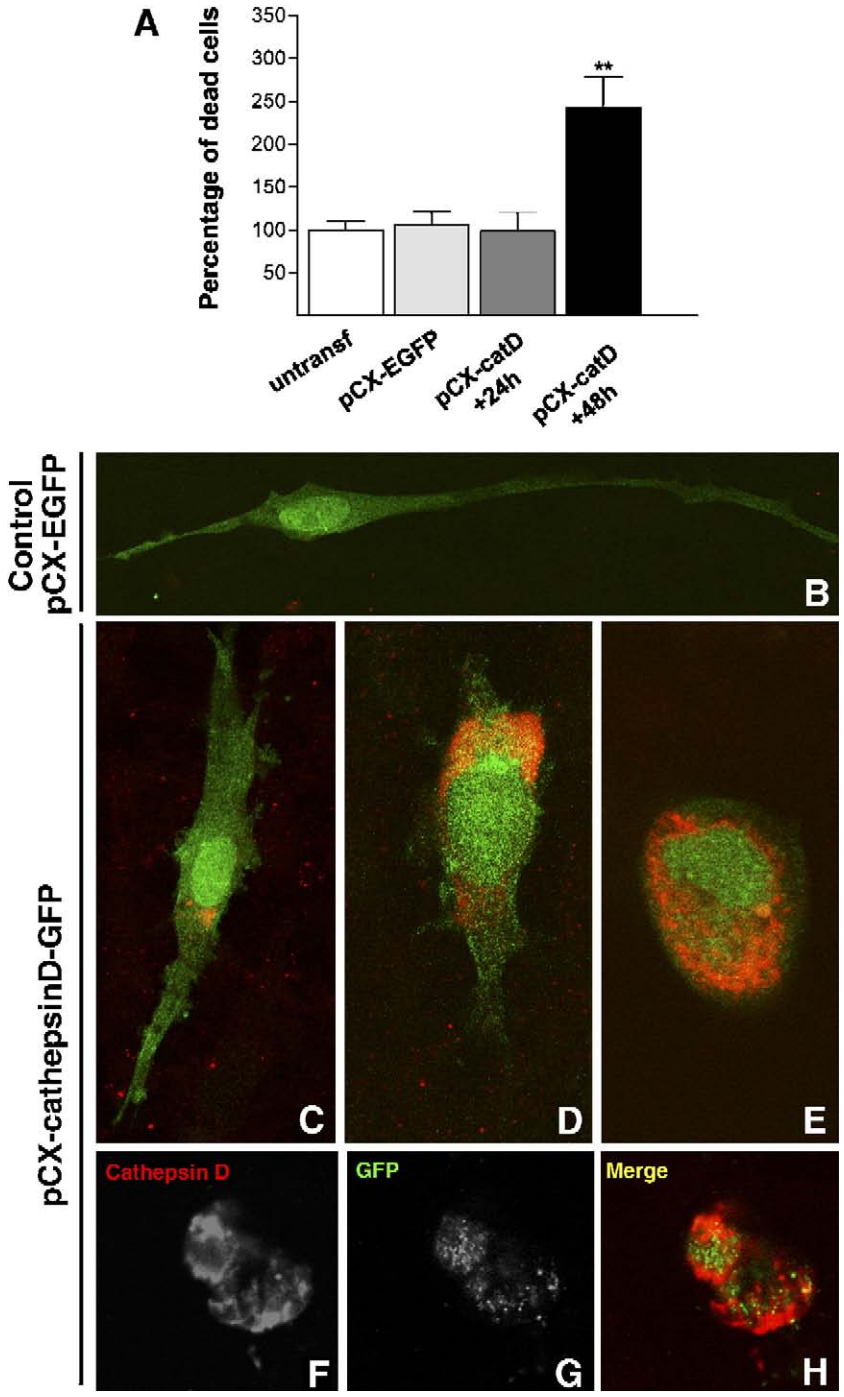

Fig. 6. Degenerative cell changes associated with *cathepsin D* overexpression. (A) Graphic representation of the number of trypan blue positive dead cells in the culture medium quantified in 5 different transfection experiments. (B) Control cells transfected with pCX-EGFP vector showing the characteristic elongated phenotype of healthy mesenchymal cells. Panels C–E illustrate transfected cells (green) cultured for 24 h (C) and 48 h (D–E) showing the progressive increase in cathepsin D immunolabeling (red) associated with the loss of the elongated cell morphology. (F–H) Transfected cells at advanced stages of degeneration showing decreased GFP labeling (green; G) in correlation with cytosolic overloading of cathepsin D (red; H). In all panels, green corresponds to GFP.

In the last decades, many studies have been devoted to analyze the molecular basis of apoptosis due to the biological and medical importance of this phenomenon. From these studies, many different apoptotic factors have been identified, and lysosomal enzymes, which were largely considered as responsible for nonspecific intracellular protein degradation associated with necrosis, have appeared as new mediators of cell death (Guicciardi et al., 2004). Analysis of cell death after treatment with lysosomotropic detergents revealed that the lysosomal induction of apoptotic versus necrotic features might be explained by differences in the intensity of lysosomal permeability (Li et al., 2000). So far, the lysosomal pathway of apoptosis was observed in cell death associated to pathologic processes, such as tumors (Foghsgaard et al., 2002),;

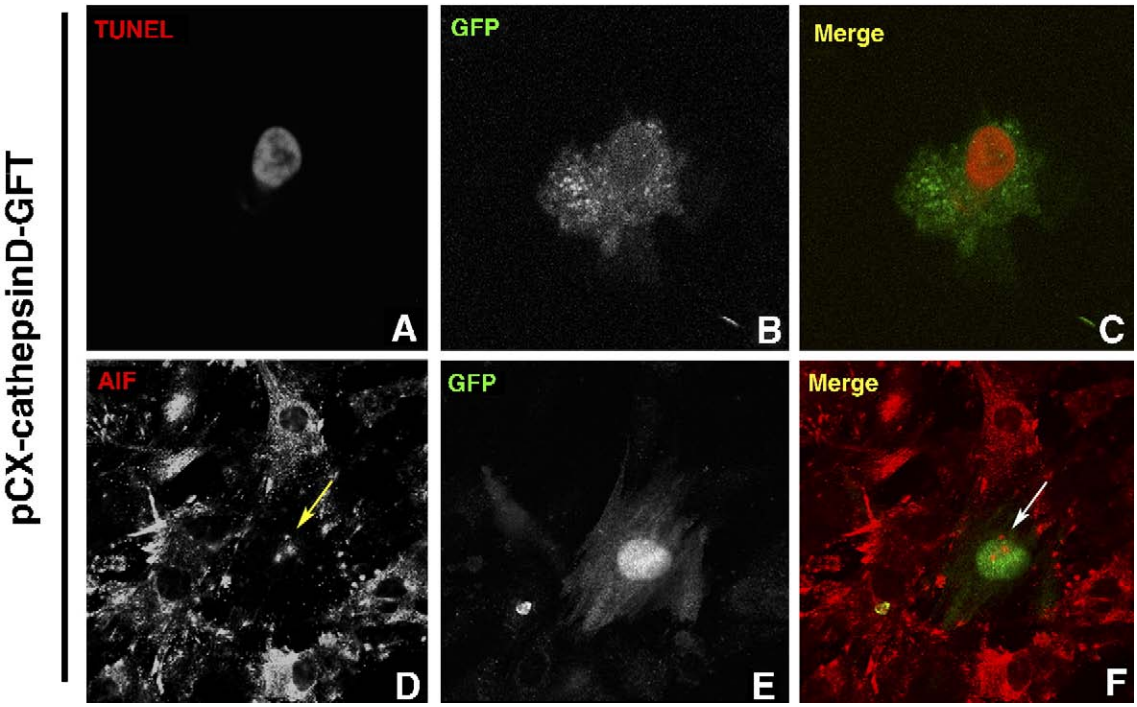

Fig. 7. TUNEL assay and nuclear translocation of AIF following *cathepsin D* overexpression. (A–C) DF1 cells transfected with pCX-cathepsin D-IG showing TUNEL positivity (red; A) in correlation with the loss of the characteristic elongated phenotype of these cells. (D–F) Immunolabeling for AIF (red; D) 48 h after transfection, showing nuclear translocation of AIF (arrow) in a pCX-cathepsin D-IG transfected cell (green; E) in contrast with the mitochondrial pattern present in neighbor cells. Note the correlation of nuclear translocation of AIF and the loss of the elongated phenotype. In all panels, green corresponds to GFP.

neurodegenerative diseases (Houseweart et al., 2003) or in cells treated with a variety of cytotoxic agents (see review by Liaudet-Coopman et al., 2005). Here, we show that *cathepsin* genes exhibit well-defined and regulated domains of expression within embryonic tissues undergoing programmed cell death instead of being ubiquitously expressed in all embryonic cells. Furthermore, overexpresion of cathepsin D is able to trigger cell death in embryonic tissues. We also show that BMPs, triggering signals for interdigital cell death (Macias et al., 1997; Merino et al., 1999), positively regulate the expression of *cathepsins D* and *B* genes in the interdigital limb bud mesoderm. And, most importantly, we show that active cathepsin D protein increases in the interdigital mesoderm prior to DNA fragmentation and is released into the cytosol at the beginning of the apoptotic process. This last finding is of considerable importance as lysosomal permeabilization constitutes a central event in apoptotic cell death mediated by lysosomes (Kagedal et al., 2005). Together, these results are consistent with a role of lysosomal proteases in programmed cell death. We have previously observed that the interdigital mesoderm of the third interdigit is not irreversibly committed to death until day 6.5 of incubation (Ganan et al., 1996). Interestingly, this stage correlates with the interdigital upregulation of cathepsin D gene and protein expression, suggesting that, as we have observed in the gain-of-function experiments, expression of this enzyme could establish the point after which cells cannot be rescued from the death program.

Consistent with the absence of the interdigital phenotype in mice singly deficient for cathepsin D, B or L (Saftig et al., 1995;

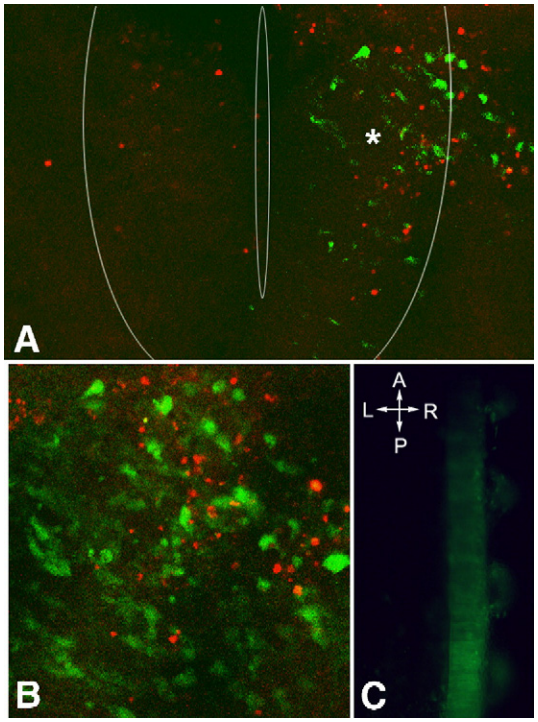

Fig. 8. *Cathepsin D* overexpression induces cell death in the neuroepithelium and somitic mesoderm. (A) Transverse section of an experimental embryo 24 h after electroporation showing cathepsin D-EGFP expressing cells in the right half of the neural tube and neighbor somite. Note the high number of TUNEL positive cells (red) in the electroporated area. (B) Magnification of the area marked with an asterisk in panel A. (C) Dorsal view of a control embryo to show the efficiency of electroporation in neural tube and adjacent somites.

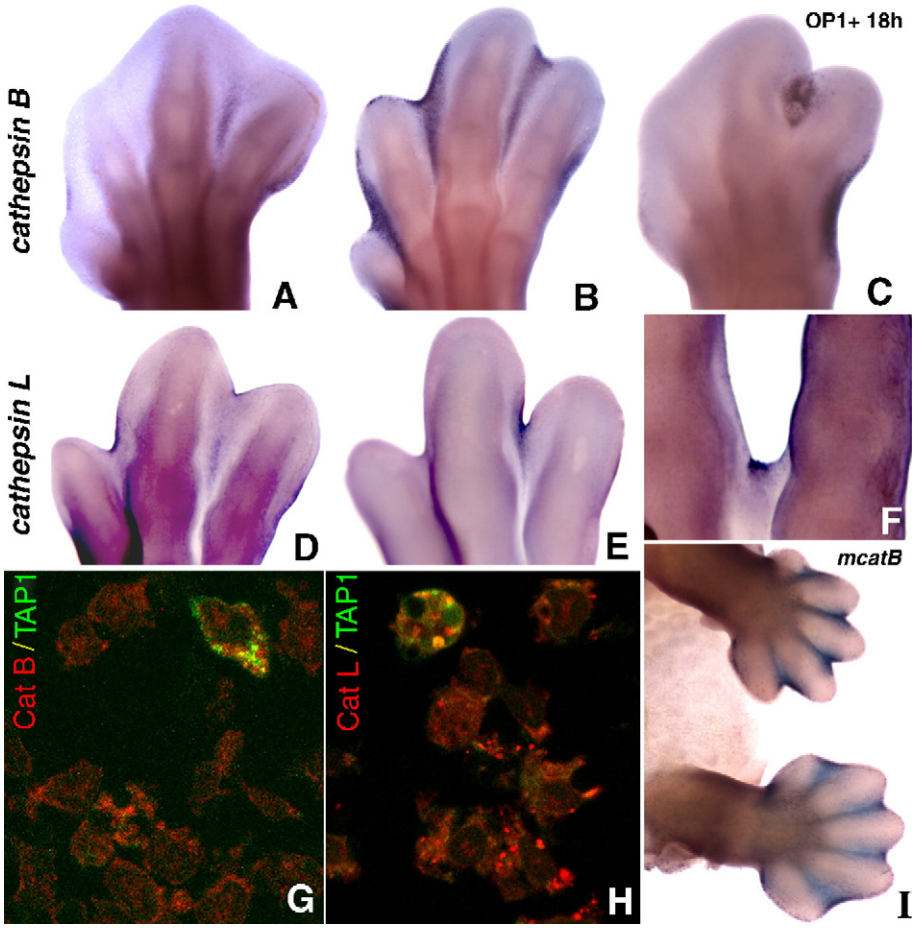

Fig. 9. Expression of cathepsin B and L in the chick and mouse interdigital regions. (A–B) Interdigital expression of *cathepsin B* at days 7.5 (A) and 8 (B) of incubation. Panel C shows the intense upregulation of *cathepsin B*, 18 h after the implantation of a BMP bead in the third interdigit. Panels D–F illustrate the late interdigital expression of *cathepsin L* in comparison with cathepsins D and B. Note the initial interdigital expression of this gene detected at day 8 (D) and its expression in the distal margin of the regressing interdigit at day 9 of incubation (F). G and H illustrate the immunolabeling of the interdigital mesodermal cells for cathepsin B (red in G) and cathepsin L (red in H) in combination with the macrophage marker TAP1 (green). Note that labeling for these cathepsins (red) appears both in the mesenchymal cells and macrophages (green). (I) *In situ* hybridization of 13.5 dpc mouse embryos, showing intense expression of *cathepsin B* in the interdigital tissue of both fore- and hindlimb.

Deussing et al., 1998; Roth et al., 2000), we have observed that these proteases would not be the only mediators of cell death in the embryonic limb. In fact, here we show that treatments with pepstatin A to inhibit cathepsin D failed to significantly inhibit interdigital cell death. However, when pepstatin A was administered in combination with the pan-caspase inhibitor Z-VAD-FMK, inhibition of cell death was intensified. It must be taken into account that Z-VAD-FMK, in addition to caspases, is also able to weakly inhibit cathepsins B and L (Rozman-Pungercar et al., 2003). These findings suggest that cathepsins and caspases cooperate synergistically in the execution of the death program.

The activation of various degenerative pathways at the same time might be a characteristic of in vivo systems undergoing massive cell death in a short period of time as occurs in the developing embryo. It must be taken into account that, at difference of in vitro systems, here degeneration includes the removal not only of mesenchymal cells but ectodermal tissue, blood vessels and the extracellular matrix (Hurle et al., 1985, 1986). Consistently with our interpretation, previous studies have shown that metaloprotease 11 might be also implicated in interdigital tissue removal (Dupe et al., 1999). However, we have unpublished evidence showing that neither calpains (1–4) nor serpin 1 is upregulated in the areas of interdigital cell death.

Evidences obtained in cell death systems mediated by cathepsin D indicate that this protease causes the permeabilization of the mitochondrial membrane (Bidere et al., 2003; Boya et al., 2003; Liaudet-Coopman et al., 2005). This effect appears to be dependent on the activation of Bax or other cytoplasmic factors (Bidere et al., 2003; Liaudet-Coopman et al., 2005) and results in the release of AIF and cytochrome *c*, which in turn induces the degradation of DNA directly or through an intermediate activation of the caspase pathways (see Jaattela et al., 2004). These findings correspond with the apoptotic mechanisms identified during interdigital cell death. We have previously shown that nuclear translocation of AIF and cytoplasmic release of cytochrome *c* are precocious events in INZ (Zuzarte-Luis et al., 2006). Additionally, a syndactylous phenotype is characteristic of mice deficient in Bax and Bak,

two important regulators of mitochondrial permeability (Lindsten et al., 2000). Indeed, here we show that nuclear translocation of AIF occurs in mesodermal cell death induced by overexpression of *cathepsin D*.

An interesting finding of our study is the observed cooperation between cathepsin D and caspases to promote interdigital cell death. This feature suggests the presence of regulatory mechanisms in the dying cells at the end of the apoptotic cascade destined to ensure its effective elimination. Indeed, we have also observed that, following caspase inhibition, the amount of cathepsin D increases in the interdigital mesoderm. These findings suggest that caspases are dispensable for the permeabilization of lysosomes, as detected in other apoptotic models (Paquet et al., 2005), and further point to the occurrence of regulatory mechanisms of compensation between both apoptotic signals.

Another potential interaction between cathepsin D and caspases and/or other proteases is the possibility that the latter facilitate the action of cathepsin D by acidifying the cytosolic pH. Several studies reported the acidification of the cytoplasm during apoptosis, and quantitative approaches provide pH values in apoptotic cells compatible with cathepsin D function (Nilsson et al., 2003).

In summary, this study provides evidences for the implication of lysosomal cathepsins in embryonic programmed cell death and for new interactions between caspases and the lysosomal enzymes during the execution of the death program.

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